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The *Heterobasidion insulare* complex from Pakistan

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Abstract—*Heterobasidion linzhiense* and *H. orientale* were collected from *Pinus wallichiana* forests. Both species are described and illustrated based on the western Himalayan specimens. Combined nrDNA ITS and LSU sequence analysis supports the identity of these species in *Heterobasidion insulare* complex. They represent new species record from pristine forested areas of Pakistan, and *Pinus wallichiana* is recorded as new host for both species.

Key words—*Bondarzewiaceae*, macrofungi, phylogeny, polypore, *Russulales*

Introduction

Heterobasidion Bref. (*Bondarzewiaceae*, *Russulales*) is a well-known polypore genus characterized by resupinate to pileate basidiocarps, a dimitic hyphal system with mostly simple septate generative hyphae and dextrinoid skeletal hyphae, and finely asperulate inamyloid and nondextrinoid basidiospores (Gilbertson & Ryvarden 1987). The genus is indigenous to many areas and an important ecological factor involved in nutrient cycling, forest regeneration, and forest succession (Garbelotto 2004, Chen & al. 2015). *Heterobasidion* has a worldwide negative impact on conifers, both ecologically and economically, by reducing site productivity and the amount of harvestable timber (Woodward & al. 1998). About twelve species have been accepted in *Heterobasidion* (Buchanan 1988, Niemelä & Korhonen 1998, Ota & al. 2006, Dai & al. 2007, Dai & Korhonen 2009, Tokuda & al. 2009, Otrosina & Garbelotto 2010, Chen

& al. 2014). Five species are currently recognized in the *H. annosum* complex: *H. abietinum* Niemelä & Korhonen, *H. annosum* (Fr.) Bref., *H. irregulare* Garbel. & Otrosina, *H. occidentale* Otrosina & Garbel., and *H. parviporum* Niemelä & Korhonen (Niemelä & Korhonen 1998; Otrosina & Garbelotto 2010). Seven species are recognized in the *H. insulare* complex: *H. amyloideum* Y.C. Dai & al., *H. australe* Y.C. Dai & Korhonen, *H. ecrustosum* Tokuda & al., *H. insulare* (Murrill) Ryvarden, *H. linzhiense*, *H. orientale,* and *H. tibeticum* Y.C. Dai & al. (Ota & al. 2006, Dai & al. 2007, Dai & Korhonen 2009, Tokuda & al. 2009, Chen & al. 2014). Most species in the *H. insulare* complex are nonpathogenic and have larger basidiospores than species in the *H. annosum* complex. Another species of *Heterobasidion* outside of these two complexes, *H. araucariae* P.K. Buchanan, occurs in Australia, New Zealand, and adjacent regions (Buchanan 1988). Previously, only *H. insulare* has been reported from Pakistan (Ahmad & al. 1997). During our present exploration of macrofungi, *H. linzhiense* and *H. orientale* were collected from coniferous forests of western Himalayas. Both morphological and molecular evidence were used to confirm the identity of these species.

Materials & methods

Morphological evaluation

Basidiomata were collected, photographed, dried, characterized morphologically, and vouchered in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH) and the Farlow Herbarium, Harvard University, Cambridge MA, USA (FH). Microscopic observations were made from slide preparations of dried specimens stained with Cotton Blue and Melzer's reagent under a Meiji MX4300H biological microscope. Anatomical features (basidiospores, basidia, cystidia, hyphae) were measured from at least 25 elements under oil immersion at 1000×; x = arithmetic mean of spore length and spore width for all spores measured. Line drawings were made with a camera lucida.

DNA extraction, PCR amplification, DNA sequencing

Genomic DNA was extracted from small pieces of basidiomata by a modified CTAB method (Bruns 1995) and using the Qiagen DNeasy Plant Mini Kit (cat. no. 69104). The internal transcribed spacer region (ITS1/5.8S/ITS2) was targeted with primer pair ITS1F/ITS4 and the large subunit (LSU) with primer pair LROR/LR5 (White & al. 1990, Gardes & Bruns 1993). PCR was carried out using Econo Taq DNA Polymerase under the following cycling parameters: initial denaturation (94 °C for 1 min), 35 elongation cycles (94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min), and final extension 72 °C (8 min). Amplified PCR products were purified and sequenced bidirectionally by Macrogen (Republic of Korea).

Table 1. Sequences of *Heterobasidion* spp. (and *Bondarzewia* outgroup) used in phylogenetic analysis. New sequences are set in bold font.

Taxon	Sample	Origin	Host	GenBank	
				ITS	nLSU
H. abietinum	00057/2	Italy	*Abies alba*	KJ651453	KJ651511
	00051/1	Italy	*Picea abies*	KJ651450	KJ651508
H. amyloideum	Li 1878	China	Unknown	KJ651455	KJ651513
	Li 1675	China	Unknown	KJ651454	KJ651512
H. annosum	09001/1	Italy	*Pinus nigra*	KJ651461	KJ651519
	K 06129/6	Russia	*Pinus sylvestris*	KJ583211	KJ583225
H. australe	K 05172/3	China	*Tsuga chinensis*	KJ651466	KJ651524
	K 05175/2	China	*Tsuga chinensis*	KJ651467	KJ651525
H. ecrustosum	K 07103/2	China	*Pinus* sp.	KJ651471	KJ651529
	K 08145/2	China	Unknown	KJ651472	KJ651530
H. linzhiense	***MSM#0097***	***Pakistan***	***Pinus wallichiana***	***MH233930***	***MH233931***
	Cui 9707	China	*Pinus* sp.	KJ651483	KJ651541
	Dai 5408	China	*Picea* sp.	KJ651484	KJ651542
H. parviporum	08129/5	Russia	*Picea abies*	KJ651501	KJ651559
	04121/3	Finland	*Picea abies*	KJ583212	KJ583226
H. orientale	**MSM#0099**	**Pakistan**	***Pinus wallichiana***	**MH233932**	**MH233933**
	K 00085/2	China	*Abies* sp.	KJ651492	KJ651550
	K 00082/6	China	Unknown	KJ651491	KJ651549
H. tibeticum	Dai 5537	China	*Pinus* sp.	KJ651507	KJ651565
	Dai 5534	China	*Pinus* sp.	KJ651506	KJ651564
B. montana	AFTOL-ID 452	—	—	DQ234539	DQ200923

Sequence alignment and phylogenetic analysis

Sequencing of the two *Heterobasidion* specimens (MSM#0098; MSM#0099) yielded fragments of 700 base pairs for ITS and 1160 base pairs for LSU. Attempted amplification of the RPB1 and RPB2 genes was unsuccessful for both taxa. Closely related ITS and LSU sequences were retrieved from GenBank based on initial BLAST results and following Chen & al. (2015). Information on all the retrieved sequences used to construct phylogenetic tree is provided in Table 1.

Sequences were manually edited and assembled using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html). Following Dentinger & al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs

5′–(…GAT)CATTA– and –GACCT(CAAA…)–3′ with the intervening alignment portion included in analysis. *Bondarzewia montana* (Pers.) Harmaja (DQ200923) was used as outgroup based on results reported by Chen & al. (2015).

Our new sequences were aligned with GenBank sequences from related taxa using ClustalX (Thompson & al. 1997) and manually edited using BioEdit (Hall 1999). Maximum Likelihood analysis was conducted using RAxML 7.2.8 (Stamatakis 2006) based on the GTR + G nucleotide substitution model. The topology was assessed by 1000 bootstrap replicates.

Taxonomy

Heterobasidion linzhiense Y.C. Dai & Korhonen,
Ann. Bot. Fenn. 44(2): 141 (2007). Fig. 1

Basidiocarp annual, pileate, sessile, solitary, leathery when fresh, corky when dry. Pileus semicircular, 4–6 cm wide, and 7.5 mm thick at base, cream buff, reddish brown at one edge, azonate; margin sharp, undulating. Pore surface cream to buff-yellow when dry; pores angular, 3–4 per mm. Tubes cream buff to buff-yellow, corky, ≤5 mm long. Odor and taste not recorded.

Hyphal system dimitic: generative hyphae without clamp connections, hyaline, occasionally branched, simple septate, thick-walled, 3–5.5 µm diam.; skeletal hyphae dominant, hyaline, rarely branched, interwoven, thick-walled with a lumen, 3.6–6 µm diam. Tubes: Generative hyphae hyaline, thin-walled, frequently simple septate, rarely branched, parallel along the tubes, 2–4.5 µm diam.; skeletal hyphae dominant, hyaline, thick-walled, occasionally branched, interwoven, 2.8–5.5 µm diam. Cystidia absent. Cystidioles present, thin-walled, hyaline, 14–24.8 × 3.2–5.8 µm; basidia clavate, four-spored, 14–19 × 5–7 µm. Basidioles similar to basidia, but smaller in size. Basidiospores broadly ellipsoid to subglobose, hyaline, finely asperulate, usually guttulate, 6–7.3 × 5.6–7 µm [x = 6.8 × 6 µm].

Material studied: **PAKISTAN**, **Khyber Pakhtunkhwa**, **Swat**, Mankial, on *Pinus wallichiana* A.B. Jacks., 5 September 2013, Malka Saba & Abdul Nasir Khalid, MSM#0097 (FH00304577, LAH310093; GenBank MH233930, MH233931).

Comments—Our Pakistani specimen is morphologically similar to the protologue description of *H. linzhiense* (Dai & al. 2007). *Heterobasidion linzhiense,* originally described from Linzhi County, Xizang (Tibet), China, is separated from the other *Heterobasidion* species by smaller pores on the undersurface of basidiomata, larger basidiospores, and subulate cystidioles (Dai & al. 2007).

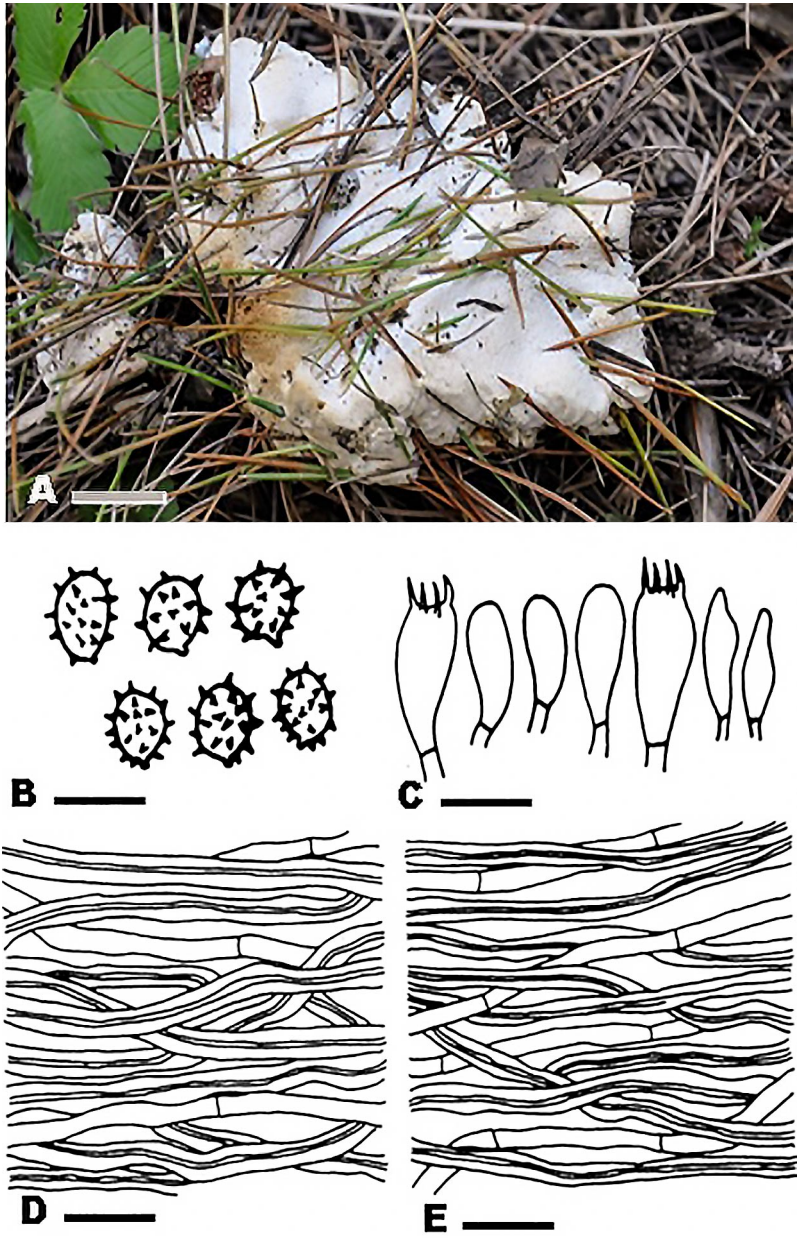

Fig. 1. *Heterobasidion linzhiense* (MSM#0097): A, Basidiomata; B, Basidiospores; C, Basidia; basidioles, and cystidioles; D, Hyphae from trama; E, Hyphae from context. Scale bars: A = 10 mm; B = 5 μm; C–E = 10 μm.

Heterobasidion orientale Tokuda, T. Hatt. & Y.C. Dai, Mycoscience 50(3): 193 (2009). Fig. 2

Basidiocarp annual, pileate, sessile, solitary, lacerate, leathery when fresh, corky when dry. Pileus semicircular, 13.5 × 2 cm, zonate, reddish brown, cream on the edge; margin sharp or blunt, undulating. Pore surface cream to buff-yellow when dry; pores round, angular or labyrinthiform, 1–4 per mm. Tubes cream buff to buff-yellow, corky, ≤8 mm long. Odor and taste not recorded.

Hyphal system dimitic: generative hyphae without clamp connections, hyaline, occasionally branched, simple septate, thick-walled; skeletal hyphae dominant, hyaline, rarely branched, interwoven, thick-walled with a lumen, 3–6.5 µm diam. Tubes: Generative hyphae hyaline, thin-walled, frequently simple septate, rarely branched, parallel along the tubes, 2–4.5 µm diam.; skeletal hyphae dominant, hyaline, thick-walled, occasionally branched, interwoven, 3–6 µm diam.. Cystidia absent. Basidia clavate, four spored, 14–26 × 7–10 µm. Basidioles similar to basidia, but smaller in size. Basidiospores globose to subglobose, hyaline, finely asperulate, 4.5–6 × 4–5 µm, [x = 5.5 × 4.3 µm].

Material studied: **PAKISTAN**, **Khyber Pakhtunkhwa**, **Shangla**, Yakh Tangay, on *Pinus wallichiana*, 2 September 2013, Malka Saba & Abdul Nasir Khalid, MSM#0099 (FH00304569, LAH310093; GenBank MH233932, MH233933).

Comments—Our Pakistani specimen is morphologically similar to the protologue description of *H. orientale* (Jang & al. 2007). *Heterobasidion orientale* is characterized by sessile to effused-reflexed annual basidiocarps covered by a thin crust, a reddish brown pileus with a marginal white zone, and larger regular to labyrinthiform pores. This species shared genetic similarity with *H. australe* and *H. tibeticum* in nrDNA sequences (Fig. 3). Morphologically, presence of large pores on the undersurface (1–4/mm), the absence of a glazed pore surface, and slightly larger spores distinguish *H. orientale* from *H. australe,* which is also excluded by the annual habit, larger pores, and absence of amyloid context hyphae (Chen & al. 2014) characterizing our Pakistani material.

Phylogeny

Our final dataset comprises twenty ingroup and one outgroup sequences, two of which were generated during this study. After removing and editing the ambiguous letters from the aligned sequences, we phylogenetically compared 1935 positions of which 1778 characters were conserved, 148 were variable, 41 were parsimony informative, and 107 were singletons.

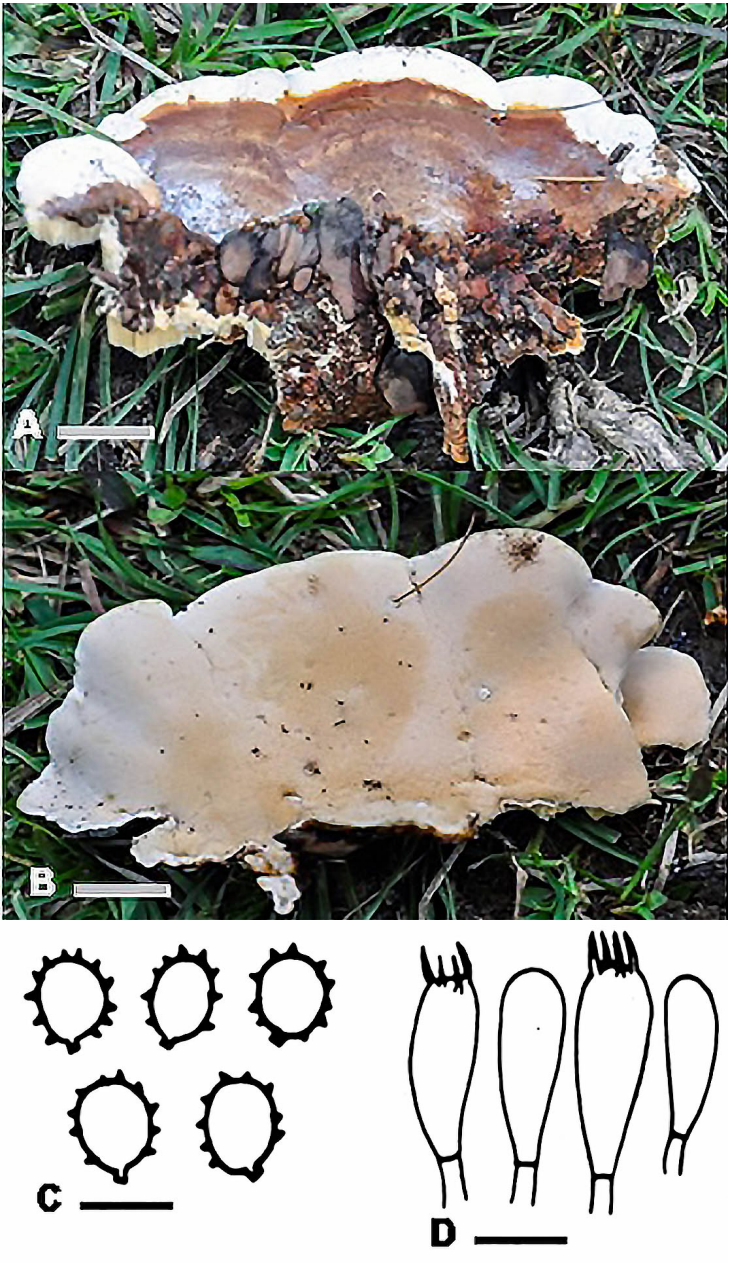

Fig. 2. *Heterobasidion orientale* (MSM#0099): A, B, Basidiomata; C, Basidiospores; D, Basidia and basidioles. Scale bars: A, B = 12 mm; C = 5 µm; D = 10 µm.

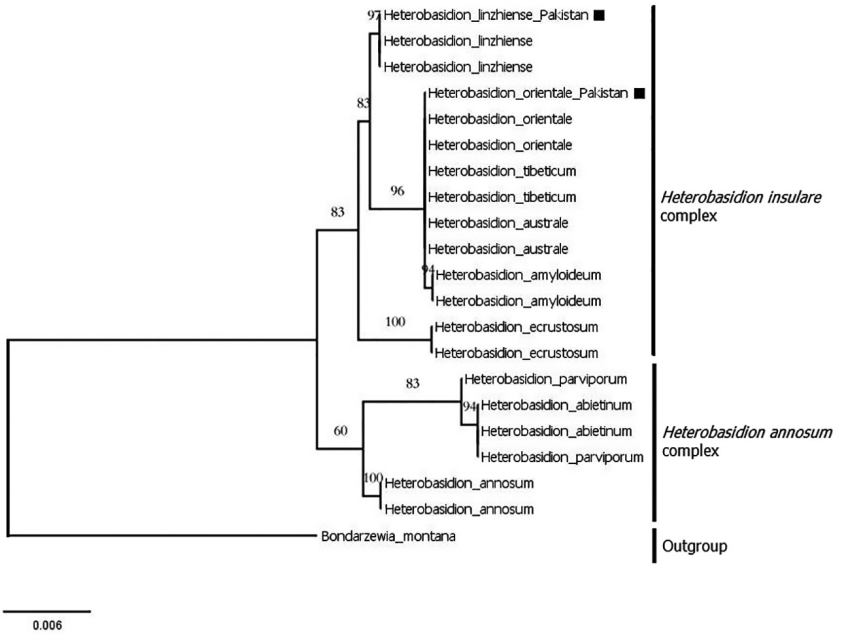

Fig. 3. Phylogenetic relationship of Pakistani *Heterobasidion* spp. and allied species based on combined dataset of nrDNA ITS + LSU sequences using maximum likelihood (ML) method. Bootstrap values >50% are cited above the nodes. Sequences generated during this study are labeled with ■.

Phylogenetic trees inferred using ITS sequences and ITS+LSU sequences showed no topological differences; we display only the combined ITS+LSU-based tree (Fig. 3). Species in the tree are divided into two *Heterobasidion* groups, the *H. insulare* complex and *H. annosum* complex, with the two new Pakistani sequences clustered in the *H. insulare* complex.

The Pakistani *H. linzhiense* sequence clustered with the other *H. linzhiense* sequences with strong (97%) bootstrap support.

The Pakistani *H. orientale* sequence clustered with sequences of *H. australe*, *H. orientale*, and *H. tibeticum* due to high genetic similarity in their rDNA sequences; we identified the Pakistani specimen based on its close morphological similarities with *H. orientale*. There are no differences in the ITS and LSU sequences among the three species. *Heterobasidion orientale* has nomenclatural priority and would be the correct name if the three species are found to be synonymous.

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